

Post-Translational Regulation of AP-1 Transcription Factor DNA-Binding Activity in the Rat Conceptus

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ABSTRACT

Activator protein-1 (AP-1) transcription factor DNA binding is induced during transient oxidative stress in the midorganogenesis rat conceptus in culture. L-2-Oxothiazolidine-4-carboxylate (OTC), a cysteine prodrug, prevented oxidative stress and the induction of AP-1 binding activity in the embryo but not in the yolk sac. Because AP-1 activity may be a significant determinant of developmental outcome after insult, we investigated the regulation of AP-1 activity in the conceptus. Supershift assays indicated that basal AP-1 binding in the embryo was due primarily to JunD, whereas in the yolk sac c-Jun and JunD were important. Under oxidative stress, c-Fos and c-Jun contributed to the AP-1 binding in the embryo; in the yolk sac, a c-Fos-shifted complex emerged. OTC protection from oxidative stress did not change the AP-1 composition, suggesting

that increased AP-1 activity was due to post-translational modifications. Changes in AP-1 activity in embryos under oxidative stress or with OTC protection were not the result of alterations in the net phosphorylation state of Fos or Jun proteins or of changes in activities of the extracellular signal-regulated kinases 1 and 2 or stress-activated protein kinases. However, immunodepletion of redox factor 1 (Ref-1), a nuclear factor that promotes AP-1 binding, eliminated AP-1 activity from embryonic nuclear extracts under both basal and oxidative stress conditions. Therefore, Ref-1 plays a critical role in regulating AP-1 activity in the conceptus; it is plausible that Ref-1-mediated modulation of the AP-1 stress response is a determinant of embryonic fate.

A broad spectrum of highly conserved cellular programs has evolved to protect cells from permanent damage or death after exposure to environmental stress (Smith and Fornace, 1996). Many perturbations, including oxidative stress, transiently induce the activity of the immediate-early transcription factor, activator protein-1 (AP-1). The immediate-early members of AP-1 include c-Fos, c-Jun, JunB, and JunD. AP-1 exists as heterodimers of Fos/Jun proteins or homodimers of Jun/Jun. In addition, Fos and Jun may form heterodimers with proteins from outside the AP-1 family (reviewed in Angel and Herrlich, 1994). The wide array of potential AP-1 conformations assures sequence specificity and transactivation potency (reviewed in Karin, 1995).

The DNA-binding and gene-activation abilities of the various AP-1 dimers are controlled by several post-translational modifications, including phosphorylation status and redox state. The phosphorylation of c-Jun on several serine/threonine residues lying adjacent to the DNA-binding domain may sterically and electrochemically hinder DNA-protein interactions (reviewed in Karin, 1995); dephosphorylation of these

residues by an unknown phosphatase is required for the binding of Jun to the AP-1 response element (reviewed in Karin, 1995). An additional mediator of AP-1 binding is the redox state of a conserved cysteine residue contained within the DNA-binding domains of Fos and Jun proteins (Abate et al., 1990). Redox factor-1 (Ref-1) reduces this moiety to its sulfhydryl form, promoting the association of Fos and Jun to DNA (Xanthoudakis and Curran, 1992).

The regulation of AP-1 transactivation may also depend on site-specific changes in phosphorylation and/or redox state. Site-specific phosphorylation of the N-terminal transactivation domains in c-Jun (reviewed in Karin, 1995) and in JunB and JunD (Sutherland et al., 1992) may result in the induction of gene activation. N-terminal phosphorylation of c-Jun is catalyzed by the stress-activated protein kinases (SAPKs; p46^{MAPK}, p54^{MAPK}; Kyriakis et al., 1994), also known as Jun N-terminal kinases (JNKs; Hibi et al., 1993; Dérjard et al., 1994); alternatively, this reaction may be mediated by the extracellular signal-regulated kinases (ERKs) 1 and 2 (Pulverer et al., 1991; Radler-Pohl et al., 1993). Homologous regulatory regions have been described for c-Fos, which is activated through the Fos-related kinase (p88^{MAPK}; Deng

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ABBREVIATIONS: AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; ERKs, extracellular signal-regulated kinases; GSH, reduced glutathione; GSSG, oxidized glutathione; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; OTC, L-2-oxothiazolidine-4-carboxylate; SAPKs, stress-activated protein kinases or Jun-activating kinases (JNKs); TBS-T, 137 mM NaCl, 20 mM Tris-HCl (pH 8), 0.1% Tween 20; DTT, DL-dithiothreitol; JAB-1, Jun-associated binding protein-1; redox factor 1.

and Karin, 1994). Redox status may also influence AP-1 transactivation ability. Phorbol ester activation of AP-1 reporter constructs was demonstrated to be Ref-1-dependent (Hirota et al., 1997).

The whole-rat embryo culture system is widely used to investigate the effects of putative teratogens (Sadler and Warner, 1984; Hales, 1991). A number of factors, including the tripeptide glutathione (L- γ -glutamyl-L-cysteinylglycine), protect the rodent embryo against stressors, including reactive oxygen species (Harris et al., 1987; reviewed in Wells et al., 1997). Glutathione exists in oxidized (GSSG) and reduced (GSH) forms. As the most abundant cellular nonprotein thiol, the GSSG:GSH ratio reflects the cellular redox balance and serves as an important measure of oxidative stress (Meister, 1976). When rat embryos were placed in culture, there was a significant increase in the GSSG:GSH ratio, peaking within 30 min and returning to baseline by 90 min (Ozoliņš and Hales, 1997, 1999). Concomitant with the increased GSSG:GSH ratio was a transient induction of *fos* and *jun* mRNAs and AP-1 DNA-binding activity (Ozoliņš and Hales, 1997, 1999). Furthermore, the culture-induced changes in GSSG:GSH ratio and AP-1 regulation were not observed in the conceptus when exogenous superoxide dismutase or catalase were added to the culture medium. The 5-oxo-L-proline analog, L-2-oxothiazolidine-4-carboxylate (OTC; 5 mM), increased GSH biosynthesis in the conceptus (Harris et al., 1987) and prevented the rise of the GSSG:GSH ratio in the embryo but not in the yolk sac (Ozoliņš and Hales, 1999). The effects of OTC on glutathione homeostasis in the embryo and yolk sac were reflected in the tissue specificity of the AP-1 response, i.e., inhibition of the induction of AP-1-binding activity in the embryo but not in the yolk sac (Ozoliņš and Hales, 1999). Together, these data support a role for glutathione homeostasis in AP-1 regulation.

Although the AP-1 response may be an important determinant of developmental outcome in response to oxidative stress, there is a relative dearth of information about the regulation of AP-1 activity in the conceptus. The purpose of these studies was to investigate AP-1 regulation in the conceptus by using OTC to protect against oxidative stress in a tissue-specific manner.

Materials and Methods

Embryo Culture. Timed-pregnant Sprague-Dawley rats (200–225 g) (Charles River Canada Ltd., St. Constant, Quebec) were housed in plastic cages with hardwood bedding and maintained in a temperature-controlled environment with a 12-h light/dark cycle. Food (Purina Rat Chow; PMI Feeds, St. Louis, MO) and tap water were provided ad libitum. On the morning of the 10th day of gestation (morning of sperm-positive vaginal smears was defined as gestational day 0), embryos were explanted from the dams and cultured according to the method of New (1978), as described (Ozoliņš and Hales, 1997). The cysteine prodrug OTC (Sigma Chemical Co., St. Louis, MO) increases glutathione biosynthesis (Williamson and Meister, 1981) and prevents oxidative stress within the conceptus (Ozoliņš and Hales, 1999). OTC was added directly to the culture medium at a final concentration of 5.0 mM; previous studies (Harris et al., 1987) have shown that this concentration increases glutathione biosynthesis in the conceptus without causing overt embryotoxicity (Ozoliņš and Hales, 1999).

To serve as a positive control for SAPK induction, some concepti were either heat-shocked or exposed to UV irradiation (Hibi et al., 1993; Kyriakis et al., 1994). Intact embryos in the culture flasks were

exposed to heat shock in a water bath at 42°C for 30 min (Kapron-Brás and Hales, 1991), returned to 37°C, and collected 30 min later. In the UV treatment paradigm, embryos and yolk sacs were separated and exposed to 100 J/m² of germicidal UV irradiation, returned to 37°C, and collected after 30 min.

Cell Culture. Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin, was used to culture HeLa and RBA (murine mammary carcinoma cells, American Type Culture Collection, Rockville, MD). As a positive control for SAPK induction, HeLa cells were exposed to 100 J/m² of germicidal UV irradiation and collected 30 to 60 min later. As a control for induction of the ERKs, RBA cells were serum-starved for 48 h in medium containing 0.1% fetal calf serum, re-exposed to medium containing 10% fetal calf serum, and collected 30 min later.

Preparation of Nuclear Extracts. Crude nuclear extracts from embryos and yolk sacs were prepared exactly as described (Ozoliņš and Hales, 1999). Nuclear extracts derived from cell cultures were prepared similarly, except that they were not sonicated in the first step.

Gel-Shift Assays. Assays were performed as described (Ozoliņš and Hales, 1997) using the DNA fragment containing the human collagenase AP-1-binding site (Angel and Karin, 1991). The concentration of DL-dithiothreitol (DTT) used in the binding buffer was determined empirically as the minimum amount needed to produce visible binding activity after overnight exposure of the autoradiograph. This varied from 0.1 to 0.5 mM DTT.

Supershift Assays. Supershift assays compared basal (0 min) AP-1 binding with the period of peak AP-1 binding (30 min). To better visualize the relative contributions of AP-1 constituent proteins, the nuclear extracts were normalized for binding activity rather than protein content. Affinity-purified rabbit polyclonal antibodies (1 μ g), specifically recognizing only c-Fos, c-Jun, phospho c-Jun, JunB, or JunD proteins (Santa Cruz Biotechnologies Inc., Santa Cruz, CA), were added to preformed AP-1/DNA complexes in the binding mixture [10 μ l of 20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 1 mM DTT, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25 mg/ml poly dI-dCl]. Naive affinity-purified rabbit IgG (1 μ g; Zymed Inc., San Francisco, CA) was used to control for nonspecific rabbit IgG interactions. After 1 h of incubation at room temperature, 10 \times loading buffer was added [250 mM Tris-HCl (pH 7.5), 40% glycerol]. Electrophoretic mobility-shift assays (EMSAs) were done in 4% native acrylamide gels (0.5 \times Tris-borate EDTA, 1% glycerol) with 0.5 \times Tris-borate EDTA running buffer. Specificity of the interaction between the antibody and the DNA-binding complex was demonstrated by the abolition of a supershift on addition of a peptide fragment containing the antibody epitope and the lack of effect after addition of a fragment that did not contain the epitope (data not shown).

Kinase Assays. Immunoprecipitations were carried out with modifications to published methods (Harlow and Lane, 1998). Concepti were sonicated or cells were lysed in 25 mM HEPES (pH 7.5), 1% Triton X-100, 1% (w/v) deoxycholate, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 3 mg/ml aprotinin, 40 mg/ml bestatin, 10 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM sodium orthovanadate, and 40 mM β -glycerophosphate (Hibi et al., 1993) using an ultrasonic processor (Sonics and Materials Inc., Danbury, CT). Homogenates were cleared by 15 min of centrifugation at 15,000g at 4°C. The protein concentrations of the soluble extracts were determined by using the method of Bradford (BioRad Laboratories Inc., Mississauga, ON) and were equalized with lysis buffer. Sample extracts (conceptus, 500 μ g; cells, 300 μ g) were first preadsorbed with 5 μ g of naive, affinity-purified rabbit IgG (Zymed Inc., San Francisco, CA), followed by a 1-h incubation with 10 μ l (50% suspension) of protein G-linked Sepharose beads (Pharmacia Biotech Inc., Baie D'Urfé, Québec) at 4°C. Samples were cleared as described above. The supernatants were removed and sequentially immunoprecipitated with rabbit affinity-purified polyclonal pan-ERK and pan-SAPK antibodies (recognizing, respectively, ERKs-1,2 [p44^{MAPK}, p42^{MAPK}] and the SAPKs [p54^{MAPK}, p46^{MAPK}];

both from Santa Cruz Biotechnologies Inc.) and cleared by a 1-h incubation at 4°C with protein G. Immunocomplexes were washed five times in lysis buffer, three times in 25 mM HEPES (pH 7.5), 0.2% Triton 100, 1 mM EDTA, and twice in kinase reaction buffer [25 mM HEPES (pH 7.5), 20 mM MgCl₂, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate (NaVaO₄), and 2 mM DTT]. Pellets were mixed with 50 μl of kinase reaction buffer, 5 μg of substrate (GST-c-Jun[1–79]; Santa Cruz Biotechnologies Inc.), 10 μCi of [³²P] ATP (6000 mmol/Ci; Amersham Canada Ltd., Oakville, Ontario). The reaction was carried out at 30°C for 15 (SAPKs) or 60 min (ERKs), because the activity of ERKs toward c-Jun is less than that of the SAPKs (Hibi et al., 1993; Kyriakis et al., 1994). The reaction was terminated by the addition of loading buffer [25 mM Tris-HCl (pH 6.8), 2% SDS, 1% glycerol, 5% 2-mercaptoethanol] and 3 min of boiling, and the products were resolved by SDS/12% polyacrylamide gel electrophoresis (PAGE). Gels were dried and autoradiographed. Analysis was done by densitometric quantification of autoradiograms (Scanmaster 3+; Howtek Inc., and Masterscan; Scanalytics Inc., Billerica, MA) and confirmed by excision of the fragment from the gel and liquid scintillation counting (LKB1217 Rackbeta; Pharmacia Biotech, Inc.).

Alkaline Phosphatase-Sensitive Mobility Shifts. The protocol used was as described (Wang et al., 1996), except that 200 μg of protein was used, and c-Fos, c-Jun, and JunD were sequentially immunoprecipitated as described above and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Canada Ltd., Laval, Quebec).

Immunodepletion Assay. The consequences of Ref-1 immunodepletion were studied with nuclear extracts from embryos that were cultured for 30 min (oxidatively stressed) or cultured and treated with OTC for 30 min (protected from oxidative stress). The nuclear extracts were normalized for binding activity, rather than protein content, to permit better visualization of the effects of Ref-1 depletion. The concentration of DTT in the binding buffer was determined empirically, as described above. To control for nonspecific IgG binding, the nuclear extracts were preadsorbed with 5 μg of affinity-purified rabbit IgG (Zymed Inc.) at 4°C for 1 h on a rotator. Immunocomplexes were removed by a subsequent 1-h incubation with 5 μl (50% suspension) of protein G-linked Sepharose beads (Pharmacia Biotechnology, Inc.). The supernatant was incubated for 1 h in the presence of rabbit polyclonal affinity-purified Ref-1 antibody (3 or 10 μg, Santa Cruz Biotechnologies, Inc.) followed by immunoprecipitation with protein G. The supernatant was used in EMSA, as described above. Specificity of the removal of Ref-1 from extracts was demonstrated by competition studies with a noncatalytic Ref-1 peptide fragment containing the antibody epitope (Santa Cruz Biotechnologies, Inc.) in which the protein concentrations were kept constant with BSA (RNase/DNase-free; Boehringer Mannheim Canada Ltd., Laval, Quebec or Pharmacia Biotechnology, Ltd.).

Western Blot Analysis. Immunoprecipitates were boiled for 5 min in sample loading buffer [62.5 mM Tris-HCl (pH 6.8), 12% glycerol, 2% SDS, 5% 2-mercaptoethanol] and fractionated with 10% PAGE (Laemmli, 1970). The proteins were transferred (Towbin et al., 1979) to Hybond polyvinylidene difluoride membranes (Amersham Canada Ltd.). Blocking was done for several hours on an orbital shaker at room temperature with 5% skim milk powder and 1% BSA fraction V (Sigma) in TBS-T [137 mM NaCl, 20 mM Tris-HCl (pH 8), 0.1% Tween 20]. The membrane was washed 3 times for 10 min in TBS-T and then incubated for 1 h at room temperature in TBS-T with 5% skim milk and 1% BSA and for 3 h with the appropriate antibody (anti-c-Fos, 1:1500; anti-c-Jun, 1:1000; anti-JunD, 1:2500; anti-Ref-1, 1:1000). After three 10-min washes, the membranes were incubated for 2 h with horseradish peroxidase-linked anti-rabbit antibody (1:2500) in TBS-T, 5% skim milk, and 1% BSA; the signals were detected using enhanced chemiluminescence (Amersham Canada Ltd., Mississauga, ON).

Statistical Analysis. Data were analyzed using Student's *t* test with the CSS (Complete Statistics System) computer program (Stat-

soft, Tulsa, OK), followed by a post hoc Tukey's test. The a priori level of significance was *P* < .05.

Results

Effects of OTC on AP-1 DNA-Binding Activity. AP-1 DNA-binding activity in the embryo was induced 4-fold by culture for 30 min, relative to 0 min (basal activity) (Fig. 1A); the addition of OTC prevented the induction of AP-1 in the embryo. In the yolk sac, AP-1 DNA-binding activity at 30 min was 3-fold higher than at 0 min; unlike in the embryo, OTC had no effect on the induction of AP-1 activity in the yolk sac (Fig. 1B).

Composition of the AP-1-Binding Complex. Supershift assays were done to determine whether differences in AP-1-binding activity between oxidatively stressed embryos and those protected with 5 mM OTC were due to changes in the AP-1 constituent proteins. Samples were obtained at 0 and 30 min after the initiation of culture; 30 min was the period of maximal oxidative stress and AP-1 binding and the time point at which OTC had its most pronounced protective effect (Ozolinš and Hales, 1999). Affinity-purified rabbit IgG did not produce a supershifted band (Fig. 2, A and B). In the embryo, at the beginning of culture (0 min), only the JunD antibody retarded migration of the bound AP-1 dimer, indicating that detectable quantities of c-Fos, c-Jun, and JunB did not contribute to the basal binding activity. When the amount of antibody added to the binding mixture was doubled from 1 to 2 μg, no novel supershifted complexes were apparent (data not shown). In addition, the proportion of existing supershifted complexes relative to the total AP-1 complex did not change, indicating that the antibodies were not limiting (data not shown). Thus, other AP-1 family members (FosB or Fra 1,2) or proteins from outside the AP-1 family were also constituents of basal AP-1 binding in the conceptus.

In untreated embryos, 30 min after the initiation of culture, c-Fos and c-Jun supershifted complexes with similar mobilities appeared; these complexes may represent a c-Fos/c-Jun heterodimer. At 30 min, the mobility of the JunD complex was faster than at the initiation of culture (*t* = 0),

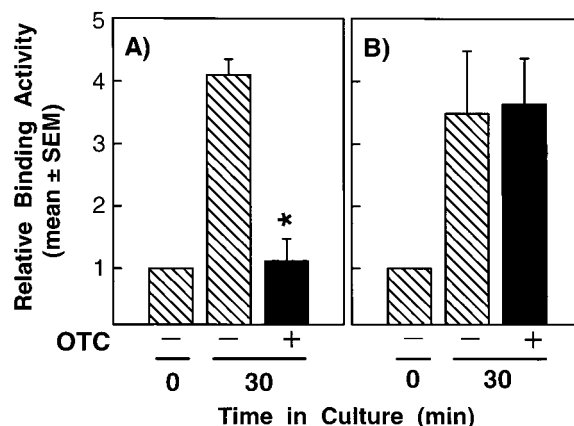


Fig. 1. The effect of OTC (5 mM) on AP-1 binding activity after 30 min of culture in the embryo (A) and the yolk sac (B). Gestational day 10.5 concepti were placed into culture for 30 min, either in the presence (+) or absence (–) of OTC. Samples were collected and separated into embryo and yolk sac, and EMSA was performed as described in *Materials and Methods*. Autoradiograms were quantified and expressed relative to basal activity (0 min). * denotes a significant difference from 30 min (–).

indicating that JunD had now dimerized to a different protein. The JunB antibody did not produce a supershifted complex, demonstrating that it did not contribute to AP-1-binding activity. OTC treatment did not further change the supershift profile. Therefore, changes in AP-1 composition were not the basis for the quantitative differences in binding between oxidatively stressed and protected embryos.

In the yolk sac, JunD was a major component of basal (0 min) binding activity, but c-Jun also contributed significantly (Fig. 2B). Similarly to the embryo, a major portion of the binding activity was from outside the scope of antibodies tested. Culture-induced oxidative stress promoted the recruitment of c-Fos into the binding complex; the relative mobilities of c-Jun and JunD were unchanged. JunB was not detected in the binding activity. Because treatment with OTC prevented neither the increase in the GSSG:GSH ratio nor the induction of AP-1 binding (Ozolinš and Hales, 1999) in the yolk sac, it was not surprising that OTC did not modify the constituent proteins in this tissue.

Phosphorylation of Jun by ERKs or SAPKs Is Not Responsible for Oxidative Stress-Induced AP-1 DNA-Binding Activity. Immunocomplex kinase assays were done to determine whether changes in the activities of the ERKs or SAPKs could account for the induction of AP-1 binding by oxidative stress. In the embryo, the activities of the ERKs at 0 and 30 min were similar, and OTC treatment did not alter these activities (Fig. 3A). Similar results were obtained in the yolk sac (Fig. 3B). Thus, in the conceptus, the increased AP-1 binding in response to culture-induced oxidative stress was not the result of the phosphorylation of Fos or Jun by the ERKs. As a positive control for induction of ERK activities, a serum starvation/re-exposure paradigm was used with RBA cells (murine mammary carcinoma cells). Relative to the serum-starved cells, the cells re-exposed to serum and collected 30 min later exhibited a 4-fold increase in ERK activities (Fig. 3, A and B).

The activities of the SAPKs were measured to ascertain their role in signaling the oxidative stress response in the

conceptus. Oxidative stress at the initiation of culture did not alter SAPK activity in the embryo; furthermore, OTC treatment had no effect on SAPK activity (Fig. 4A). Concepti or HeLa cells exposed to UV irradiation or heat shock, both potent stimuli of SAPK activities in other models (Kyriakis et al., 1994), served as positive controls. The SAPKs were refractory to UV irradiation in the embryo but were induced 4-fold by heat shock, indicating that the induction of these kinases at this stage of development is stimulus-specific. In HeLa cells, 30 min after UV irradiation, the activity of SAPKs was induced 6-fold over that of untreated cells (Fig. 4, A and B). In the yolk sac, as in the embryo, the SAPKs were not induced after perturbation with culture, and OTC had no effect (Fig. 4B). However, in the yolk sac, unlike in the embryo, both UV irradiation and heat shock induced SAPK activities, 4- and 6-fold, respectively (Fig. 4B). Therefore, conditions of oxidative stress did not alter SAPK activities. SAPKs were not involved in the induction of AP-1 DNA-binding activity, although they were inducible in a tissue- and stimulus-specific manner.

Phosphorylation Changes in Conceptual AP-1 Constituents. Because AP-1 binding and transactivation activity may depend on the balance between phosphorylation and dephosphorylation reactions, we investigated whether the phosphorylation states of c-Fos, c-Jun, and JunD changed in response to culture in a manner that was independent of the ERKs and SAPKs. JunB was not examined because it was not detected as a constituent of the AP-1-binding activity (Fig. 2). In the embryo, alkaline phosphatase-sensitive SDS/PAGE mobility shifts were not observed for c-Fos or JunD, indicating that these proteins were not significantly phosphorylated in either the basal or induced state (Fig. 5A). To further investigate the role of AP-1 phosphorylation, a supershift assay was done to determine whether transcriptionally active phospho-c-Jun was a component of the AP-1-binding complex. No supershifted phosphorylated c-Jun complex was noted in embryonic samples under basal conditions or 30 min after heat shock or oxidative stress (Fig. 6A). Under basal conditions, phosphorylated c-Jun was not detected in HeLa cell nuclear extracts, whereas a supershifted complex was detected in extracts obtained from HeLa cells 30 min after

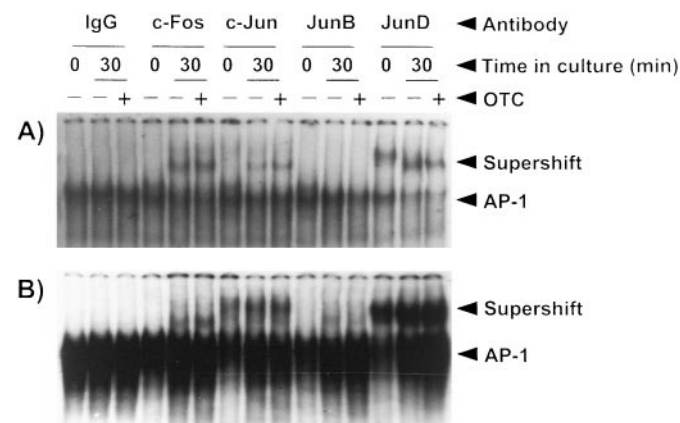


Fig. 2. Supershift assays depict the relative contributions of Fos and Jun proteins to the AP-1-binding activity in the embryo (A) and the yolk sac (B). Concepti were cultured for 30 min either in the presence (+) or absence (-) of 5 mM OTC. Samples were separated into embryo proper and yolk sac and prepared as described. The additions of naive rabbit antibodies (IgG) and others directed against Fos and Jun proteins were done as outlined in *Materials and Methods*. Nuclear extracts were normalized for binding activity rather than protein content. The AP-1 and the supershifted complexes are indicated with arrowheads. Duplicate experiments were performed and yielded similar results.

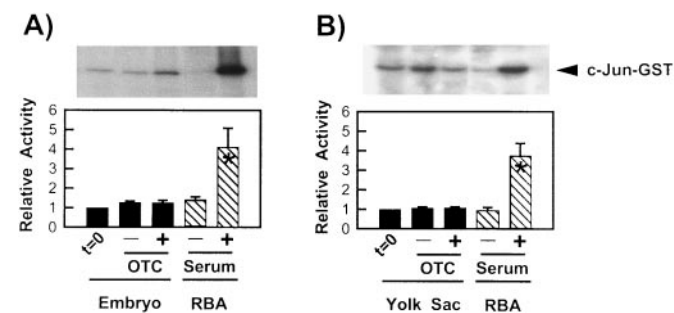


Fig. 3. The activities of the ERKs in embryos (A) and yolk sacs (B) in the presence (+) or absence (-) of 5 mM OTC. Concepti were cultured for 30 min as described in *Materials and Methods*. As a control for ERK induction, RBA cells were either serum-starved (-) or collected 30 min after the re-exposure to serum (+). Immunocomplex assays were performed in the presence of radiolabeled ATP using a recombinant c-Jun-glutathione S-transferase fusion protein as substrate. The reaction products were separated by SDS-PAGE, the gel was dried, and the incorporation of phosphate into c-Jun was detected with autoradiography. Four autoradiograms were quantified and expressed relative to 0 min. * denotes a significant difference from 0 min ($P < .05$).

UV irradiation (Fig. 6A). Thus, AP-1 binding in the embryo was not influenced by changes in the overall phosphorylation of c-Fos, c-Jun, or JunD.

The yolk sac did display alkaline phosphatase-sensitive mobility shifts for both c-Fos and JunD (Fig. 5B). There were no discernible differences between the mobilities of the 30-min stressed and the 30-min OTC-protected proteins. Because region-specific phosphorylation changes may occur even when the overall phosphorylation status is constant, it is not clear whether this form of post-translational modification is important in the up-regulation of AP-1 binding. Although a phospho-c-Jun supershifted AP-1 complex was not observed in the yolk sac extracts after oxidative stress or under basal conditions, a retarded complex was observed in samples after heat shock (Fig. 6B). Thus, c-Jun phosphorylation changes may play a role in the yolk sac in response to specific stressors.

AP-1-Binding Activity in the Embryo Is Ref-1-Dependent. To determine whether Ref-1 was the redox activity-controlling AP-1 binding in the embryo during oxidative stress, nuclear extracts were immunodepleted of Ref-1 before

running the EMSA, and the effect on binding was assessed. The yolk sac was not examined because OTC had no effect on AP-1 binding activity in this tissue. Western blot analysis confirmed the presence of Ref-1 in the immunoprecipitates of extracts treated with 10 μ g of the Ref-1 antibody (Fig. 7A); longer exposures were needed to reveal the Ref-1 signal when 3 μ g of antibody were used (data not shown). The secondary antibody detected the anti-Ref-1 antibody in samples to which protein G was added (Fig. 7, A and B). The removal of Ref-1 from the embryonic nuclear extracts with 3 μ g of antibody partially inhibited binding (Fig. 7C), suggesting that small amounts of Ref-1 are critical to AP-1 binding. Binding activity was entirely inhibited with 10 μ g of anti-Ref-1 antibody (Fig. 7C). These effects were noted in untreated and OTC-treated samples collected 30 min after the initiation of culture. Furthermore, when a constant protein concentration was maintained with BSA supplementation, the addition of the noncatalytic epitope containing Ref-1 peptide fragment restored binding activity. To address the possibility that immunoprecipitates of Ref-1 coprecipitated with the entire AP-1 binding complex, Western blots were also probed with anti-JunD antibody (the most abundant identifiable component of the AP-1 complex; Fig. 2). Although the antibody did detect a positive control peptide adsorbed to the membrane, no JunD bands were detected in the Ref-1 immunoprecipitates (Fig. 7B). Thus, the anti-ref-1 antibody did not coprecipitate the AP-1-binding complex, suggesting that the absence of Ref-1 was the cause for reduced AP-1-binding activity. These results implicate Ref-1 in the regulation of basal and inducible AP-1 activity in the embryo.

Discussion

AP-1 induction is potentially a cytoprotective response in the midorganogenesis conceptus and may be a significant determinant of developmental outcome after perturbation. The induction of AP-1-binding activity was observed in the rodent conceptus after the initiation of culture, coincident with a transient oxidative stress response. This stress response was characterized by a disruption of glutathione homeostasis, as assessed by an increase in the GSSG:GSH ratio and increased protein and DNA oxidation products (Winn and Wells, 1995; Ozolinš and Hales, 1997, 1999). Hyperoxic culture conditions also induced the activities of several antioxidant enzyme, including the glutathione peroxidases and glutathione *S*-transferases (Ishibashi et al., 1997; Ozolinš and Hales, 1999). Although the relevance of this transitory oxidative stress response to the developmental outcome of a cultured organogenesis-stage embryo remains unclear, it is significant that free radical-mediated disturbances in gluta-

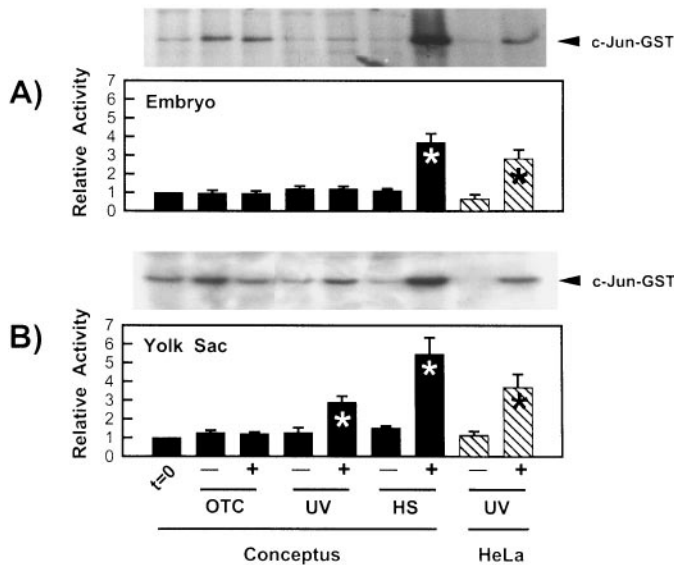


Fig. 4. The activities of the SAPKs in embryos (A) and yolk sacs (B) in the presence (+) and absence (-) of 5 mM OTC. As positive controls for the inducibility of the SAPKs, the concepti and HeLa cells were treated with UV irradiation or heat shock (HS) as described. Immunocomplex assays were performed in the presence of radiolabeled ATP using a recombinant c-Jun-glutathione *S*-transferase fusion protein as substrate. The reaction products were separated by SDS/PAGE, the gels were dried, and the incorporation of phosphate into c-Jun detected with autoradiography. Four autoradiograms were quantified and expressed relative to 0 min. * denotes a significant difference from 0 min.

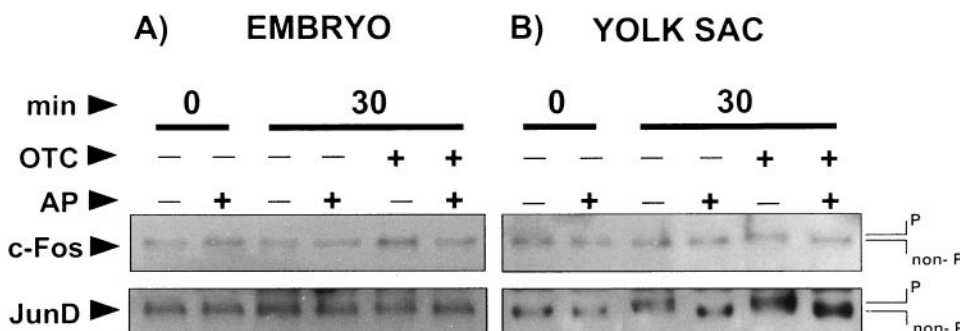


Fig. 5. The alkaline phosphatase-sensitive mobility shifts of c-Fos and JunD in embryos (A) and yolk sacs (B) in the presence (+) or absence (-) of OTC. c-Fos and JunD were sequentially immunoprecipitated from 200 μ g of embryo and yolk sac whole-cell extract and incubated in the presence (+) or absence (-) of calf intestinal alkaline phosphatase (AP). The phosphorylated (P) and nonphosphorylated (non-P) states are indicated. Three experiments showed similar results.

thione homeostasis and oxidative damage to embryonic DNA and other macromolecules have been implicated in the teratogenicity of drugs such as thalidomide (Parman et al., 1999).

The differences in AP-1 regulation between the embryo and the yolk sac in response to OTC exposure were related to the mechanism by which OTC alters glutathione homeostasis (Ozolinš and Hales, 1999). OTC increased GSH content in the embryo but not in the yolk sac. Interestingly, protein concentrations of γ -glutamyl-cysteine synthetase, as assessed in Western blots, were significantly higher in the yolk sac than

in the embryo. We can speculate that feedback inhibition of glutathione synthesis may occur in the yolk sac but not in the embryo. Other investigators, using different embryo culture conditions, have reported that OTC is more effective at maintaining glutathione homeostasis under stress in the yolk sac than in the embryo (Harris et al., 1987).

Dimeric composition is an influential determinant of the AP-1 binding and transactivational potential (reviewed in Karin, 1995). Interestingly, in the embryo, the AP-1 complex was void of c-Fos and c-Jun activity, but JunD and an unidentified nonsupershifted complex contributed significantly under basal conditions (0 min; Fig. 2A). The yolk sac differed from the embryo in that c-Jun was detected during basal conditions. Thus, JunD may be critical for the execution of developmental programs during organogenesis; in the embryo, c-Jun may not be important, whereas in the yolk sac during this period of development, c-Fos may be irrelevant. These suggestions are supported by the observation that *c-fos*-null mice were viable at birth (Johnson et al., 1992; Wang et al., 1992), whereas *c-jun*-null mice died relatively late in gestation, during the fetal period (Johnson et al., 1993; Hilberg et al., 1993). The role of JunD during development is unclear because *junD*-null transgenic mice have not yet been reported.

Curiously, in both the embryo and the yolk sac, approximately half of the basal and induced AP-1 DNA-binding activity resided in the nonsupershifted complex (Fig. 2, A and B). This complex may include other members of the AP-1 family, namely FosB, Δ FosB or Fra's 1,2. In addition, other bZIP proteins from outside the AP-1 family may also form heterodimers with AP-1. These may include ATF-2, CREBII, Maf and Nrl, MafB, NfBp65, or NfBp50 (reviewed in Angel and Herrlich, 1994). Ironically, if FosB or Fra proteins do not contribute to the unidentified components of the AP-1-binding complex, this may suggest that AP-1-dependent transcription may, in fact, be relatively AP-1-independent.

Irrespective of OTC treatment, no additional supershifted bands were observed in embryonic extracts collected 30 min after the initiation of culture. In the yolk sac, a substantial c-Fos supershifted complex was detected in addition to the c-Jun and JunD containing complexes that were present during basal conditions (0 min). The different mobilities of the c-Fos- and c-Jun-containing complexes in the yolk sac indicated that, unlike the embryo, these were not Fos/Jun heterodimers. The relatively constant overall amounts of Fos and Jun proteins in the nuclei of concepti from all three treatment groups (Fig. 2) showed that the increased binding activities were not the result of increased protein synthesis.

The implications of the large contribution of JunD to the AP-1-binding activity of the embryo and the yolk sac are unknown, although the presence of JunD strongly influences protein associations that modulate the binding of the AP-1 complex. For instance, the Jun-associated binding protein-1 (JAB-1) selectively enhances the occupancy of the AP-1 response element with dimers that contain c-Jun and JunD, but not JunB (Claret et al., 1996). Thus, although purely speculative, the differences in AP-1-binding between oxidatively stressed and protected embryos may be the result of interactions with proteins such as JAB-1; however, the role of JAB-1 during development and the stress response has not been determined.

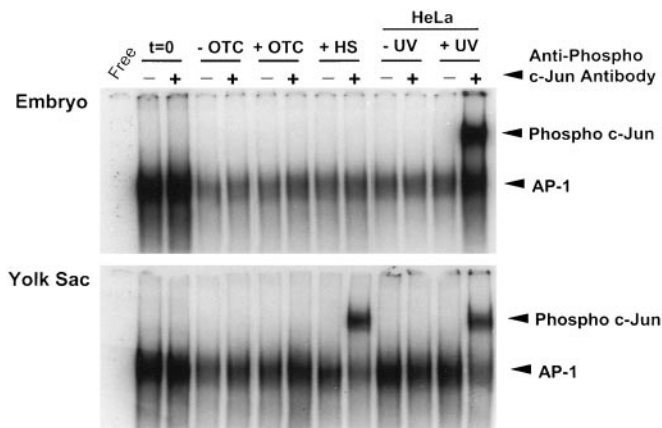


Fig. 6. Supershift assays depict the contributions of phospho-c-Jun to AP-1 binding activity in embryos and yolk sacs. Concepti were cultured for 30 min either in the presence (+) or absence (–) of 5 mM OTC or exposed to heat shock as described in *Materials and Methods*. Nuclear extracts were prepared, and samples were normalized for binding activity, rather than protein content, and incubated either in the presence (+) or absence (–) of the antiphospho-c-Jun antibody. The AP-1 and supershifted complexes are indicated with arrowheads. Duplicate experiments were done, yielding similar results.

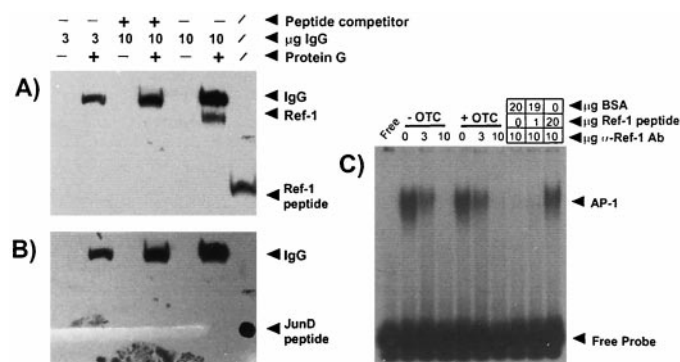


Fig. 7. The effect of Ref-1 on the AP-1 DNA-binding activity in the embryo. The Western blot in A was probed for Ref-1 and depicts the ability of different concentrations of anti-Ref-1 antibody to immunoprecipitate Ref-1 from 25 μ g of embryonic extract (A). Nuclear extracts were treated with 3 or 10 μ g of anti-ref-1 antibody with (+) or without (–) 20 μ g of competitor peptide. Samples were incubated in the presence (+) or absence (–) of 5 μ l of a 50% suspension of Sepharose-linked Protein G. After centrifugation, the precipitates were separated by SDS/12% PAGE. The antiRef-1 antibody, the Ref-1 protein, and the competitor Ref-1 peptide are identified. The Western blot in A was reprobed with an anti-JunD antibody (B). The circular dot is a positive control peptide fragment adsorbed to the membrane. EMSA was done after the immunodepletion of Ref-1 from nuclear extracts with 3 or 10 μ g of anti-Ref-1 antibody (C). Samples from the embryos cultured in the absence (–) or presence (+) of OTC were normalized for DNA binding activity (20–60 μ g of protein, respectively). Protein content was held constant with BSA as the antibody-Ref-1 interaction was blocked by competition with the addition of increasing quantities of noncatalytic Ref-1 peptide fragment. The free probe and the AP-1 complex are indicated with arrowheads.

Interestingly, JunB was not detected in the AP-1 DNA-binding activity (Fig. 2, A and B) or in the immunoprecipitates of the nuclear fraction (data not shown), even though expression of its transcripts was induced by culture of the conceptus (Ozolinš and Hales, 1999). The ramifications of the absence of JunB are unclear, owing in part to the sequence specificity of its effects on transcription (reviewed in Karin, 1995). Recently, JunB has been implicated as an important constituent of AP-1-binding activity in an in vivo carbon tetrachloride-induced hepatic model of metabolic oxidative stress; in vitro phosphorylation of JunB by SAPKs is reported to be an important part of this response (Mendelson et al., 1996). Thus, the absence of JunB may be detrimental to the conceptus when confronted with different stressors. The activities of both the ERKs and SAPKs were refractory to the oxidative stress of culture (Figs. 3 and 4). This is consistent with a report that showed that the inducibility of ERKs by H_2O_2 was tissue-specific (Kyriakis et al., 1994). Interestingly, SAPK activities were induced in the conceptus by stimuli other than oxidative stress. Treatment with UV irradiation or a dysmorphogenic dose of heat shock significantly stimulated SAPK activity in the yolk sac, although only heat shock was effective in the embryo (Fig. 4). Phospho-c-Jun was only detected in the yolk sac, despite the activation of SAPKs in both the embryo and the yolk sac in response to heat shock. First, this suggests that the SAPK kinase pathways may be poorly developed in the embryo. Second, it indicates that other stress-response proteins, such as ATF-2, may be alternative targets of heat shock-induced SAPKs (reviewed in Angel and Herrlich, 1994). Interestingly, ATF-2 does bind, albeit with different avidity, to AP-1 and AP-1 binding sequences, suggesting that ATF-2 may be responsible for increased "AP-1" binding activity (van Dam et al., 1995). The relative insensitivity of the SAPK pathway in the conceptus may contribute to sensitizing the conceptus to insult because SAPKs are thought to promote cell survival (Devary et al., 1992). In addition, mitogen-activated protein kinase kinase 4, the upstream regulator of SAPKs, may be repressed until its developmental role during hepatogenesis has been fulfilled (Yang et al., 1997).

The absence of Fos- and JunD phosphatase-sensitive mobility shifts during PAGE provided further evidence to negate the importance of phosphorylation changes in the regulation of AP-1-binding in the embryo. A single phosphorylated band was noted for both c-Fos and JunD. It has been proposed that Fos and Jun must undergo phosphorylation changes to become transcriptionally active (reviewed in Karin, 1995). Without changes in the phosphorylation state of Fos or Jun, it is not clear whether the increased AP-1 DNA-binding activity that was noted is related to a change in transcriptional activity.

The redox-regulated protein, Ref-1, promotes the DNA-protein interactions of several transcription factors, including AP-1 (Xanthoudakis and Curran, 1992; Xanthoudakis et al., 1992). Previous studies have demonstrated that the Ref-1 protein can be detected immunohistochemically in the rat fetus (Wilson et al., 1996). The immunodepletion of Ref-1 from nuclear extracts and the ensuing abolition of AP-1-binding activity demonstrate, for the first time, the absolute requirement for Ref-1 for in vitro AP-1-binding function in the embryo (Fig. 7). A functional requirement for Ref-1 in early embryogenesis is supported by the preimplantation death of mouse embryos lacking the *ref-1* gene (Xan-

thoudakis et al., 1996). However, because of the bifunctionality of Ref-1, it is not known if embryonic death is the result of improper gene expression by Ref-1-dependent transcription factors or the accumulation of oxidative DNA lesions caused by the absence of Ref-1 endonuclease activity (Xanthoudakis et al., 1996).

The activities of antioxidant enzymes in the embryo are extremely low compared with adult values (reviewed in Wells et al., 1997); consequently, the cysteine residues of Fos and Jun that control redox-dependent binding may be particularly sensitive to oxidative stress (Abate et al., 1990). This may explain why oxidatively stressed and OTC-protected samples were equally dependent on Ref-1 for the protein-DNA interaction. Ref-1 may be required at all times for AP-1 binding, not just during oxidative stress.

It is difficult to determine whether Ref-1 redox activity is induced in response to oxidative stress and whether this contributes to increased AP-1 binding and transactivation in oxidatively stressed embryos. Recent data demonstrate that, after stimulation with a phorbol ester, thioredoxin moves from the cytosol into the nucleus and physically associates with Ref-1 (Hirota et al., 1997). Because of the concomitant increase in the expression of AP-1-driven reporter constructs and the requirement for the thioredoxin catalytic center, it has been suggested that the thioredoxin/Ref-1 association induces Ref-1 redox activity, which, in turn, stimulates AP-1 transactivation. Therefore, the inducibility of Ref-1 may be an important component in the initiation of the AP-1 response, particularly in the embryo, where AP-1 phosphorylation changes are not apparent. Interestingly, Ref-1-dependent inhibition of gene expression also has been reported (Okazaki et al., 1994).

The generation and persistence of oxidative DNA damage in rodent embryos as a result of culture and exposure to teratogens (Winn and Wells, 1995) suggest that Ref-1 may be an important component of embryonic DNA repair during organogenesis. Ref-1 may have two roles in the embryonic oxidative stress response. It may promote the AP-1 dependent changes in gene expression necessary for survival in a hyperoxic environment, and it may initiate the repair of oxidative DNA damage.

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